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Award Number: W81XWH-06-1-0690

TITLE:
Identification of New Serum Biomarkers for Early Breast Cancer Diagnosis and Prognosis Using Lipid Microarrays

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REPORT DATE:
September 2008

TYPE OF REPORT:
Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-09-2008	FINAL ADDENDUM	1 SEP 2006 - 31 AUG 2008

4. TITLE AND SUBTITLE

Identification of New Serum Biomarkers for Early Breast Cancer Diagnosis and Prognosis Using Lipid Microarrays.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0690

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Stony Brook University
West 5510 Frank Melville Memorial Library
Stony Brook, NY 11794-0002

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Breast cancer is the most common form of cancer among women. Compared with other serum polypeptides, autoantibodies have many appealing features as biomarkers including sensitivity, stability, and easy detection. Anti-lipid autoantibodies are routinely used in the diagnosis of autoimmune disease, but their potential for cancer diagnosis has not been explored. Metabolism of lipids immediately follows cellular stimulation, resulting in various lipid metabolites. Dysregulation of cellular signaling in cancer cells would be expected to lead to irregular metabolism of many lipids, which could be sensed by immune system and cause the production of novel autoantibodies. Our hypothesis is that a broad and irregular change in lipid profiles in breast cancer cells results in the production of anti-lipid antibodies that could be used as biomarkers for early diagnosis. We have generated arrayed lipids on the membranes (lipid microarrays), and developed a new fluorescent detection method, which would allow us to better compare the serum reactivity to lipids. However, no serum response difference is observed in the breast cancer mice. We conclude that the current lipid microarray is not suitable for breast cancer diagnosis and prognosis yet.

15. SUBJECT TERMS

Breast cancer, serum biomarker, lipid microarrays, transgenic mouse model.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	8	USAMRMC

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Introduction:

Breast cancer is the most common form of cancer among women. Compared with other serum polypeptides, autoantibodies have many appealing features as biomarkers including sensitivity, stability, and easy detection. Anti-lipid autoantibodies are routinely used in the diagnosis of autoimmune disease, but their potential for cancer diagnosis has not been explored. Metabolism of lipids immediately follows cellular stimulation, resulting in various lipid metabolites. Dysregulation of cellular signaling in cancer cells would be expected to lead to irregular metabolism of many lipids, which could be sensed by immune system and cause the production of novel autoantibodies. Indeed, recent reports describe anti-lipid antibody production in cancer patients. Our hypothesis is that a broad and irregular change in lipid profiles in breast cancer cells results in the production of anti-lipid antibodies that could be used as biomarkers for early diagnosis. We propose to generate arrayed lipids on the membranes (lipid microarrays), and use them to examine global anti-lipid profiles at different stages of carcinogenesis in a transgenic breast cancer model.

This is a high-risk proposal. However, the relevance to breast cancer is high and the potential reward is great. If the proposed experiments are successful, anti-lipid autoantibodies will be used as a new type of biomarker that could be used in conjunction with other biomarkers for early breast cancer diagnosis. Knowledge of the lipid changes would also help us understand breast cancer pathogenesis, and might thus lead to the design of new therapeutic strategies.

Body:

We have successfully set up the technology for lipid microarrays in our laboratory. Using fluorescently labeled secondary antibodies and an internal control labeled with a different fluorophor, we have greatly improved the sensitivity and reproducibility compared to the first generation of lipid microarrays. We are currently collecting more serum from newly bred mice at different ages. The major progresses are summarized below.

Improvement of currently existing lipid microarray methodology

In the original lipid array protocol, chemiluminescent detection was used to detect antibody reactivities to lipids and glycolipids spotted on PVDF membranes. Chemiluminescent detection relies on an enzymatic reaction that produces light, which is detected by a CCD camera or imaged on film. Some samples produce bright light for a short time, and others produce comparatively dim light, but for a long period of time. Therefore, images must be collected at an optimized time. This time-dependence of signal compromises quantification and accuracy. We decided to improve this technique by using a dual-labeled fluorescent detection, which can be detected by a LI-COR Odyssey instrument. The first two issues we set up to test were the membrane support and blotting solution. The PVDF membrane was used to spot lipids in the original lipid arrays. However, autofluorescence was consistently high on PVDF membrane when the fluorescently labeled secondary antibodies were used. We then decided to test if other membranes could lower the autofluorescent background. Nitrocellulose membrane had low background, but reacted to some solvents used to dissolve lipids (data not shown). We then chose to use a new type of PVDF from Amersham, Hybond-LFP, which the manufacturer claimed to have low fluorescent background on Western blotting. We compared the performance of regular PVDF and HyBond-LFP in three different blocking buffers. Different amounts of asialo-GM1 (200 pmol, 40 pmol, 8 pmol, 3.2 pmol and 0.64 pmol) were spotted on either Hybond-LFP or regular PVDF (BioRad). The membranes were blocked in BSA, casein, or Odyssey (from LI-COR) blocking buffers, detected by a polyclonal rabbit asialo-GM1 antibody and followed by IRDye 800 goat anti-rabbit (green channel) secondary antibody. Although no secondary antibodies were used for channel 700, the background in this channel is extremely high on PVDF membranes in all three blocking conditions (Fig 1. Right panel). However, the background in channel 700

is minimal on Hybond-LFP (Fig 1. left panel). The background in channel 800 is low and is very similar in all blocking solutions on both regular PVDF and Hybond-LFP. Among all blocking solutions, Odyssey blocking buffer gave the best sensitivity (Fig 1. bottom, left panel).

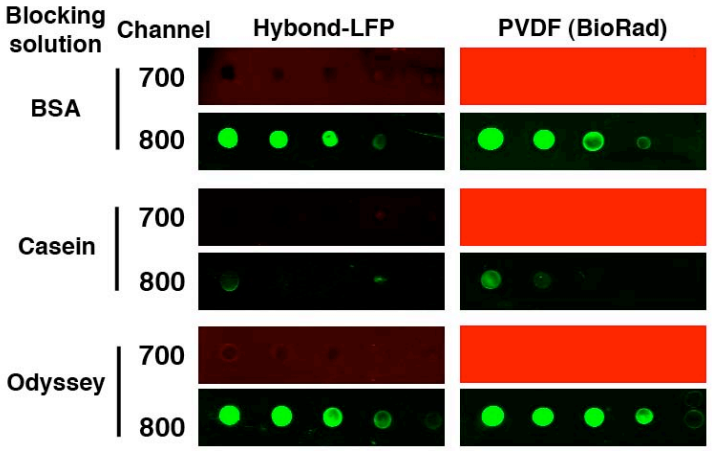

Fig 1. Improvement of lipid array methodology. Asialo-GM1 was serially diluted (200 pmol, 40 pmol, 8 pmol, 3.2 pmol and 0.64 pmol) and spotted on either Hybond-LFP or regular PVDF (BioRad). The membranes were blocked in BSA, casein, or Odyssey (from LI-COR) blocking buffers, then detected by a polyclonal rabbit asialo-GM1 antibody and followed by IRDye 800 goat anti-rabbit secondary antibody. The membranes were then scanned in both channel 700 (red) and channel 800 (green) using a LI-COR Odyssey instrument.

Array validation

We have validated our lipid arrays using polyclonal and monoclonal antibodies with defined specificities. The polyclonal antibodies specific for asialo-GM1 bound specifically to GM1, but not to the closely related gangliosides GM1 or GM2 (Fig. 2). Monoclonal antibodies raised against GD3 specifically bound GD3, but not to asialo-GM1, GM1 and GM2 (Fig. 2). The secondary antibodies did not show reactivity against lipids (data not shown).

Asialo GM1	GM1
GM2	GD3

Fig 2. Lipids on the PVDF membrane can be detected by specific antibodies. 200 pmol of Asiao-GM1, GM1, GM2 or GD3 were each spotted on PVDF membrane. The membrane were then blocked in Odyssey blocking buffers, incubated with rabbit asialo-GM1 polyclonal and mouse monoclonal GD3 antibodies, and detected by Alexa 680 goat anti-mouse (red channel) and IRDye 800 goat anti-rabbit (green channel) secondary antibodies. Asiao-GM1 and GD3 antibodies have reactivity only to the corresponding lipids. No non-specific signal to irrelevant lipids was detected.

The specificity of lipid array and dual-color fluorescent labeling should allow us to label mouse serum samples using one color, and a well-characterized polyclonal rabbit antibody against an lipid (such as asialo-GM1) using another color simultaneously. The reactivity to asialo-GM1 can then be used as an internal control, allowing us to compare different membranes, and thus providing a better method to quantitate fluorescent intensity on different membranes.

Mice breeding and serum collection

We successfully expanded our mice and started to collect serum from both wild-type control and transgenic breast cancer mice control. We collected serum from mice and used them for the arrays we generated as shown in Figure 3.

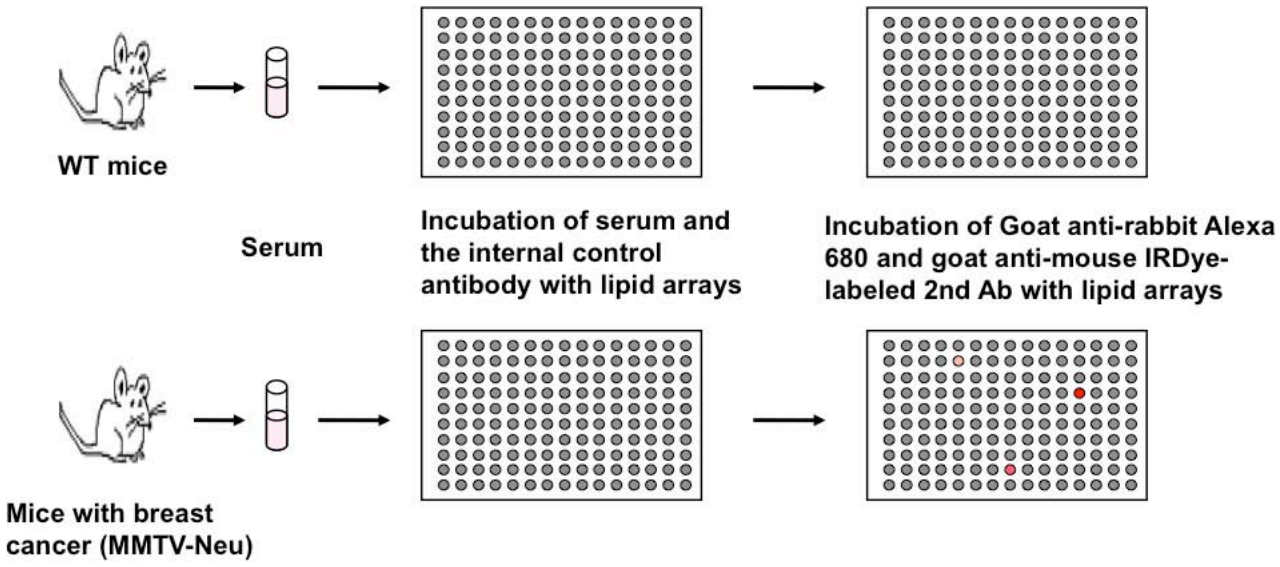

Fig 3. Steps of detecting anti-lipid serum responses. Specific signals appear in the transgenic breast mice would be the potential biomarkers for breast cancer diagnosis and prognosis. 1. Sera are collected from the FVB/N wild-type and MMTV-Neu transgenic mice. 2. Membranes spotted with different lipids are incubated with mouse sera and rabbit polyclonal Asialo-GM1 antibody (internal control). 3. The membranes were then detected with Alexa 680-conjugated goat anti-rabbit and IRDye 800-conjugated goat anti-mouse antibodies.

We had expected that we would get some novel findings when this project was finished. However, we could not detect the difference of anti-lipid antibodies for lipids we have tried in serum from normal can cancer mice (Fig 4A and B), although we have greatly improved the current methodology. We conclude that at the current technology, lipid microarray is not a preferred method for anti-lipid antibody detection in breast cancer diagnosis and prognosis.

GM1	GM2	GD3	GD1a	PS	POPA	L-α PA	14:0 PC
18:1 PI(4,5)P2	18:1 PI(3,5)P2	liver PI	PGPC	16:0-09:0(ALDO)PC	azPC Ester	Lyso Egg PC	POPC
18:1 PI(3,4,5)P3	LPA	DAG	16:0-18:1 DG	16:0 CDP DG	Cardiolipin	MLCL	sphingosine
Lactosylceramide	Cerebrosides	08:0 Ceramide-1-PO4	Ceramides	C_8Ceramide	Lyso Sphingomyelin	Sphingomyelin	Sphingosine-1-PO4, S1P
Glucocerebrosides	Cholesterol CH	PAF	Squalene	Prostaglandin E2	Prostaglandin D2, PGD2		
Asialo GM1(200 pmol)	Asialo GM1 (40 pmol)	Asialo GM1 (8 pmol)	Asialo GM1 (1.6 pmol)	Asialo GM1 (0.32 pmol)			

Fig 4A. Lipids spotted on Hybond-LFP membrane. 200 pmol of various lipids are each spotted on membrane. The last row is spotted with serious dilutions of Asialo-GM1, which are used as an internal control for different membranes.

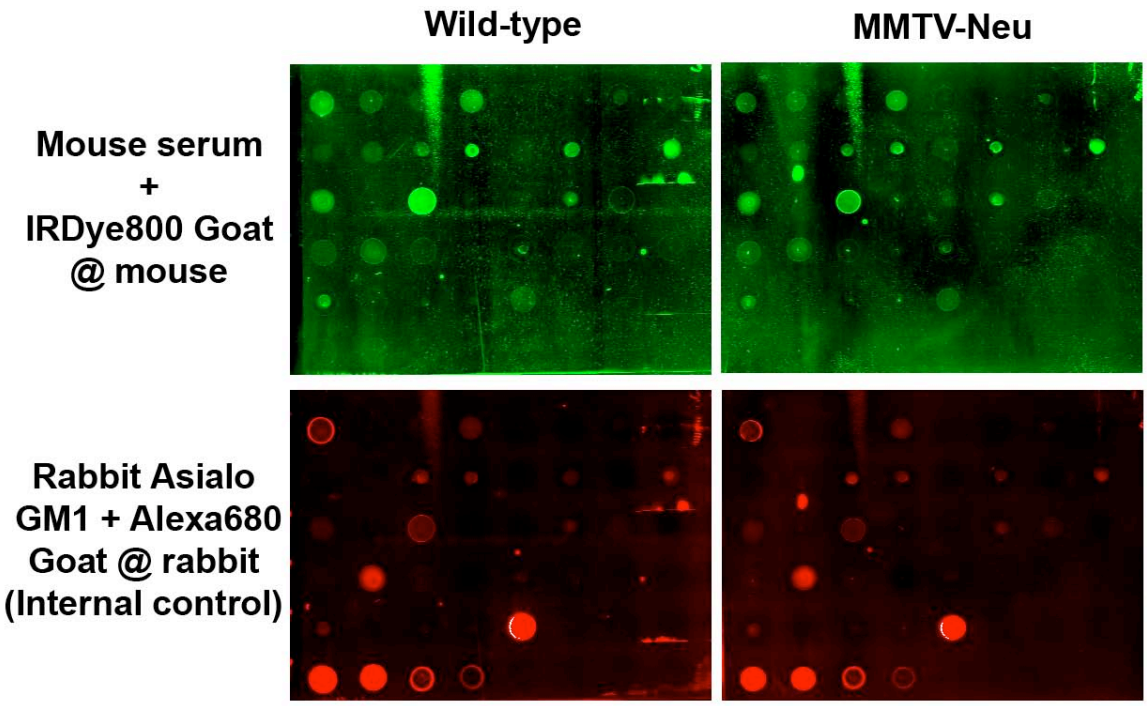

Fig 4B. No differences in serum responses to lipids between wild-type and MMTV-Neu mice. Sera collected from the FVB/N wild-type and MMTV-Neu transgenic mice and rabbit polyclonal Asialo-GM1 antibody were incubated with the membranes spotted with different lipids. The membranes were then detected with Alexa 680-conjugated goat anti-rabbit (red channel) and IRDye 800-conjugated goat anti-mouse (green channel) antibodies. The anti-Asialo-GM1 intensity is used as an internal control for different membranes.

Key Research Accomplishments

- Set up a new lipid arrays methodology based on fluorescent detection. This change improved accuracy and dynamic range of lipid arrays.
- Validated the feasibility of lipid arrays
- Bred and expanded transgenic mice.
- Collected some serum samples from mice.
- Profiled the anti-lipid serum responses in normal and breast cancer mice.
- Concluded that the current lipid microarray is not suitable for breast cancer diagnosis and prognosis.

Reportable Outcomes

The funding has been used to support part of PI's and a technician (Yue Zeng)'s salaries. It has also provided research training for the technician, who is now enrolled in the medical school of Indiana University. The breast mouse model we used in this study will be also used in the other breast cancer research involved in the lipid signaling.

We have presented our results on a poster in the 5th Era of Hope meeting at Baltimore Convention Center, Baltimore, Maryland (June 25-28, 2008). The abstract is listed below:

Characterization of serum biomarkers for early breast cancer diagnosis and prognosis using lipid microarrays.

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Breast cancer is the most common form of cancer among women. Compared with other serum polypeptides, autoantibodies have many appealing features as biomarkers including sensitivity, stability, and easy detection. Anti-lipid autoantibodies are routinely used in the diagnosis of autoimmune disease, but their potential for cancer diagnosis has not been explored. Metabolism of lipids immediately follows cellular stimulation, resulting in various lipid metabolites. Dysregulation of cellular signaling in cancer cells would be expected to lead to irregular metabolism of many lipids, which could be sensed by immune system and cause the production of novel autoantibodies. Indeed, recent reports describe anti-lipid antibody production in cancer patients. Our hypothesis is that a broad and irregular change in lipid profiles in breast cancer cells results in the production of anti-lipid antibodies that could be used as biomarkers for early diagnosis. We propose to generate arrayed lipids on the membranes (lipid microarrays), and use them to examine global anti-lipid profiles at different stages of carcinogenesis in a transgenic breast cancer model.

Conclusion

The new fluorescent detection would allow us to better compare the serum reactivity to lipids. However, the current lipid microarray is not suitable for breast cancer diagnosis and prognosis yet.

References

In preparation.

Appendices

None

Supporting data

All figures and legends were included in the main body.